치의학박사 학위논문

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Zhu Tingting의 치의학박사 학위논문을 인준함

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Abstract

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Introduction:

Thymosin β_4 ($T\beta_4$), with 43 amino acids, is a major G-actin sequestering protein, which was originally isolated from bovine thymus. In previous studies, exogenous $T\beta_4$ was demonstrated to have multifunctional activities such as angiogenesis, anti-apoptosis, anti-oxidative stress and anti-inflammation.

Dimethyloxalylglycine (DMOG) was reported to inhibit oxygen-dependent degradation of hypoxia inducible factor-1 alpha (HIF-1α). The stabilization of HIF-1α can lead to up-regulation of angiogenesis markers, which favor wound healing. Previous study demonstrated that DMOG could enhance the production of vascular endothelial growth factor (VEGF) in periodontal fibroblasts. However, there is no information yet about the effects of $T\beta_4$ and

DMOG on wound healing in the oral mucosa. To investigate the feasibility $T\beta_4$ and DMOG as oral wound healing agents, in this study, the effects of the molecules on cell motility, mRNA expression of matrix metalloproteinases (MMPs), mRNA and protein expression of angiogenesis marker genes and wound closure of rat palatal were observed.

Materials and Methods:

Rat palatal (RP) cells obtained from Sprague-Dawley (SD) rats were established in primary culture. Migration assay was performed by using a culture-insert. Matrix metalloproteinase 2 (MMP2) and VEGF were chosen to demonstrate whether the mRNA and protein expression of both markers were influenced by treatment with $T\beta_4$ at various concentrations (0, 1, 10, 100, 1000 ng/ml) and at different time courses (6 hours and 24 hours). Additionally, the mRNA and protein expressions of VEGF of RP cells treated with DMOG at different concentrations (0, 0.1, 0.5, 1, 2 mM) were evaluated. To confirm the stabilization effect of DMOG on HIF-1α, the protein expression of HIF-1α was detected by western blotting. For the *in vivo* assay, excisional wounds, 3 mm in diameter, were made at the center

part of the palate of the SD rats. $T\beta_4$ and DMOG with vehicles were topically applied 3 times during one week. The wound areas were measured photographically and histologically at 1 week after preparation of wounds.

Results:

$T\beta_4$ stimulated RP cell migration at the higher concentrations (100 and 1000 ng/ml) in 0% FBS level while there was no effect of DMOG on the migration of RP cells. $T\beta_4$ increased the mRNA expression and protein level of both the MMP2 and VEGF significantly. $T\beta_4$ enhanced the VEGF mRNA expression time-dependently. $T\beta_4$ stimulated the MMP2 mRNA level at 6 hours significantly but it decreased the expression of MMP2 mRNA at 24 hours. In addition, the mRNA and protein expressions of VEGF were significantly stimulated by the treatment of DMOG at higher concentrations. HIF-1α was stabilized in protein level by the treatment with DMOG. In animal study, it was found that 1 mg/ml $T\beta_4$-treated and DMOG-treated groups increased the rat palatal wound contracture significantly.

Conclusions:

According to the present study, both $T\beta_4$ and DMOG enhanced the wound healing of rat palatal mucosa. Therefore, $T\beta_4$ and DMOG can be considered

as the candidates for agents promoting wound healing of oral mucosa.

Keywords: wound healing; oral mucosa; thymosin β_4; dimethyloxalylglycine

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Chapter I.

Background and literature review

Wound healing process

In all cases, when tissue injury occurs, tissue integrity is re-established through a wound healing process which mainly consists of four phases: haemostatic phase, inflammatory phase, proliferative phase and remodeling phase. The wound healing can be completely fulfilled only if the four phases are properly experienced. Although the healing process is similar, the rate of healing is dependent on location, size, infection, age and overall health of the host.

- Haemostatic phase

Hemostasis is immediately initiated by tissue injury. The defect region is covered by the forming fibrin clot which gradually dries out to become a scab. The fibrin clot consists of collagen, platelets, thrombin and fibronectin to stop blood loss after vascular injury and limit the spread of pathogens into the blood stream (Davie EW, Fujikawa K *et al*. 1991).

Platelets are aggregate together with the release of a number of signaling

molecules such as platelet derived growth factor (PDGF) (Martin P. 1997; Baumgartner HR, Hosang M. 1988), transforming growth factor β (TGF-β) (Martin P. 1997; Assoian RK, Komoriya A *et al*. 1983), vascular endothelial growth factor (VEGF) (Möhle R, Green D *et al*. 1997), interleukin 1 alpha (IL-1α) (Broughton G 2nd, Janis JE *et al*. 2006) and tumor necrosis factor alpha (TNF-α) (Broughton G 2nd, Janis JE *et al*. 2006). These platelet derived molecules are responsible for the chemotactic recruitment of circulating inflammatory cells like neutrophils, monocytes and macrophages into the wound site, which gives in turn rise to inflammation - the next stage in healing.

- Inflammatory phase

Inflammatory phase parallels the haemostatic phase and occurs after injury for hours to days. The aim of this phase is to control or eliminate the injury. Neutrophils are the first type of white blood cell to be attracted into the wounds, arriving within a few hours of injury (Martin P. 1997; Gillitzer R and Goebeler M. 2011). Neutrophils clean the wound site as they kill invading bacteria and release proteases before they are removed via phagocytosis (Martin P and Leibovich SJ. 2005). Within 2-3 days after injury neutrophils are replaced by monocytes which are recruited from the

blood. They are drawn to the site of injury by release of chemoattractants released by activated endothelial cells (Shukaliak JA and Dorovini-Zis K. 2000; Engelhardt E, Toksoy A *et al*. 1998). During inflammation monocytes differentiate into macrophages. Macrophages are a heterogeneous population, as they can be involved in inflammation (M1-macrophages) but also switch to an anti-inflammatory "M2a phenotype" (Mantovani A, Biswas SK, *et al*. 2013; Novak ML and Koh TJ. 2013). Specifically, macrophages can remove debris, initiate an immune response via their antigen-presenting properties and secrete a large number of growth factors such as fibroblast growth factor (FGF) (Anderson JM. 2001; DiPietro LA. 1995). Those factors can further activate keratinocytes and fibroblasts to repair the wounds (Martin P. 1997; Anderson JM. 2001). If wound decontamination is finished, several mechanisms operate to limit and down regulate inflammation (Henson PM. 2005). In this phase, localized heat, swelling, erythema, discomfort and functional disturbance could occur (Tortora GJ and Grabowski SR, 1996).

The primarily catabolic inflammatory process is transient, but crucial for the following steps of the anabolic phase of proliferative phase.

- Proliferative phase

During this phase, the wound is filled with newly formed connective tissue

by a combination of granulation, contraction and epithelialization. Three kinds of cells, endothelial cells, fibroblast cells and epithelial cells, involve in this process.

1) The formation of granulation tissue:

Granulation tissue is a kind of highly vascularized tissue, which consist of collagen and an extracellular material called ground substance. These provide the scaffolding where new capillaries will grow to form connective tissue. Endothelial cells are required for the formation of new capillaries, which can be derived from endothelial cells of the original blood vessels as well as from the circulating endothelial progenitors (Potente M, Gerhardt H *et al*. 2011). The growth of new blood vessels is termed angiogenesis. Macrophages produce various substances to stimulate angiogenesis, for example: transforming growth factor (TGF), which enhances the formation of new tissue and blood vessels (Nathan CF. 1987).

2) Wound contraction

After the formation of connective tissue, fibroblasts congregate around the wound margin to pull the wound's edges together called wound contraction. The fibroblast cells are from monocyte-derived fibrocytes, vessel-derived

pericytes and are possible from a process termed epithelial-mesenchymal transition (Grieb G, Steffens G *et al*. 2011; Reilkoff RA *et al*. 2011; Weber CE *et al*. 2012). This kind of process favors the healing of large and open wounds (Brown GL, Curtsinger LJ *et al*. 1988).

3) Re-epithelialisation

The re-growth of epithelial cells across the wound surface occurs during the final stage of proliferation. A humid wound environment increases the process, allowing epithelial cells to migrate easily (Winter GD. 1962). The process of epithelial migration is significantly slowed in the presence of necrotic tissue or a scab. Epithelialisation happens when granulation tissue fills the wound defects. Epithelial cells originate from the keratinocytes at the wound edges. But in the case of skin, stem cells of the hair follicle can also contribute to the re-epithelialization (Blanpain C and Fuchs E. 2009; Cordeiro JV and Jacinto A. 2013)

- Remodeling phase

The remodeling phase aims to restore skin integrity. In this phase, disorganized fibers are bundled, cross-linked and aligned along tension lines. Cells such as most of the neutrophils, endothelial cells, macrophages and

myofibroblasts are no longer needed. Instead, angiogenesis diminishes and blood vessels mature to be used as functional network, which leads the wound to demonstrate its pink color and to become progressively paler (Greenhalgh DG. 1998).

Matrix metalloproteinases (MMPs)，secreted by fibroblasts, macrophages and endothelial cells, play an important role in remodeling phase. Their function is to degrade a number of extracellular matrix molecules, such as collagen and fibronectin. In addition, MMPs are known to involve in the release of apoptotic ligands, like FAS ligand. MMPs are controlled by different cytokines (TGF-β, PDGF, IL-1α, EGF). The inhibitors of metalloproteinases (TIMP), which is up-regulated by IL-6 and TNF-α (Henry G and Garner WL. 2003), suppress MMP activity.

Scar tissue, also termed fibrosis, is formed due to the various pathologies linked with inflammation. However, the pathophysiology of fibrosis is still not known (Meneghin A and Hogaboam CM. 2007). Therefore, great effort should be taken to avoid the formation of scar (Wynn TA and Ramalingam TR. 2012).

Compare oral wound healing with general skin wound healing

Although wound healing process of oral mucosa is similar to that of skin, a minimal or completely scarless repair was shown in oral wound healing. In a pig model study, wounded oral mucosa exhibited reduced scar formation compared with skin incisions (Wong JW, Gallant-Behm C *et al*. 2009).

Oral wounds also showed accelerated healing compared with skin wounds, which is demonstrated in several studies (Sciubba JJ, Waterhouse JP *et al*. 1978; Szpaderska AM, Zuckerman JD *et al*. 2003).

Bussi M reported that skin transposed into the oral cavity maintained its own morphology, especially epithelia sustain its original characters (Bussi M, Valente G *et al*. 1995). Intraoral keloids occurred on grafted skin (Reilly JS, Behringer WH *et al*. 1980). These research results indicated that intrinsic characteristics of oral mucosal tissue may be the key factors. Additionally, environmental factors such as temperature, salivary flow, are also associated to oral mucosa repair. For instance, periodontal wound healing was ended up with dense epithelial connective tissue grafts, which is supposed to offer long-term stability of the area (Thoma DS, Hämmerle CH *et al*. 2011; Santagata M, Guariniello L *et al*. 2012). Palatal wound healing is delayed in desalivated rats and larger wounds are more sensitive to desalivation than smaller wounds (Bodner L, Dayan D *et al*. 1993)

Interestingly, interleukin-1 (IL-1), which is highly up-regulated during the inflammatory phase, only exists in oral wound healing. The reason may be that IL-1 is necessary to take part in defence mechanisms against commensal bacteria in the oral cavity (Graves DT, Nooh N *et al*. 2001). Specifically, when injury occurs, pre-stored IL-1 is immediately released by keratinocytes, acting and calling for neutrophils to the damaged site (Barrientos S, Stojadinovic O *et al*. 2008). In addition, neutrophils further spur the inflammatory response to achieve by recruiting monocytes, supporting macrophage at the wound site.

Up to now, deeply observation will be requested to understand the mechanisms about the less-scar and faster healing of oral wound. The aspects unique to the oral wound healing may be the key target for the research.

Therapeutic agents for oral wound healing

In terms of theory, the therapeutic methods, which can affect the wound healing process, can be used as a kind of therapeutic regimen. In the case of oral wound, the common methods like bandages to protect wounds cannot be

used for oral wounds. In addition, the environment of the oral cavity with a relatively large commensal flora and the stimulation from mastication can worsen the oral wound healing. Therefore, the agents for protecting the wound and accelerating the wound healing process are urgently needed.

There are some commonly used drugs on oral wound healing. Non-steroidal anti-inflammatory drugs (NSAIDs) class such as ibuprofen and colchicines affect inflammatory phase. Prednisolone, belongs to corticosteroids, has an effect on haemostatic phase, inflammatory phase and remodeling phase. Disinfectants such as chlorhexidine or chlorine dioxide affect inflammatory phase (Cleland WP. 2001; Enoch S, Grey JE *et al*. 2006). The effect of chlorhexidine (Perio.Kin®) on the oral wound healing was studied by a rat model (Hammad HM, Hammad MM *et al*. 2011). However, there are some side effects about these drugs such as bacterial resistance, discoloration and dysgeusia (Gjermo P. 1989).

In contrast, natural products, such as honey, tea tree oil and potato peeling, have more healing effects and fewer side effects. Therefore, it may be a trend to cure oral wound healing by using safe, natural and effective ingredients. Many efforts should be made to develop new therapeutic agents.

Jansen RG *et al* have found collagen scaffolds accelerated the

myofibroblasts and blood vessels ingrowth in the palate of rats (Jansen RG, van Kuppevelt TH *et al*. 2008).

Previously, TGF-β1, PDGF, FGF have been studied as oral wound healing agents (El Gazaerly H, Elbardisey DM *et al*. 2013; Marx RE, Carlson ER *et al*. 1998; Oda Y, Kaqami H *et al*. 2004). Because the growth factors are expensive, easily degraded and hard to handle, the agent which has the similar effects with these growth factors and meanwhile can overcome those shortcomings is in great request.

The purpose of the present study was to evaluate the effects of thymosin β_4 ($T\beta_4$) and DMOG on the oral wound healing in order to test the therapeutic potential of these two agents for oral wound healing.

Chapter II.

Effects of thymosin β_4 on wound healing of rat palatal mucosa

2.1 Introduction

Thymosin β_4 ($T\beta_4$), an oligopeptide consisting of 43 amino acids, is known to sequester G-actin monomers. Up-regulation of $T\beta_4$ by cDNA-mediated transfection was found to cause actin depolymerization in fibroblast cells (Sanders MC, Goldstein AL *et al*. 1992), which demonstrated that modulation of $T\beta_4$ affected the polymerization state of actin cytoskeleton, as well as other cellular processes related to the organization of actin cytoskeleton, such as cell migration. Ito M *et al*. (Ito M, Iguchi K *et al*. 2009) showed that overexpression of $T\beta_4$ enhanced the formation of actin-based pseudopodia and cell motility in prostate cancer cells, providing direct evidence that $T\beta_4$ plays a role in cell migration. Colon cancer cells overexpressing $T\beta_4$ also exhibited enhanced cell migration, due in part to activation of the Rac1 signaling pathway (Tang MC, Chan LC *et al*. 2011).

In addition, extracellularly administered $T\beta_4$ promoted cell migration of various types of cells including cardiomyocytes, human umbilical vein endothelial cells and conjunctival epithelial cells (Bock-Marquette I, Saxena A *et al*. 2004; Malinda KM, Goldstein AL *et al*. 1997; Sosne G, Hafeez S *et al*. 2002). The mechanism by which exogenous $T\beta_4$ influences cell migration is not yet completely understood. However, a previous study showed that $T\beta_4$ was rapidly internalized by cells, which indicates the involvement of an intracellular receptor in the effects of the peptide (Cierniewski CS, Sobierajska K *et al*. 2012). Besides the effect on cell migration, exogenous $T\beta_4$ was also found to have multifunctional activities such as angiogenesis, anti-apoptosis, anti-oxidative stress and anti-inflammation (Grant DS, Rose W *et al*. 1999; Reti R, Kwon E *et al*. 2008; Kumar S and Gupta S. 2011; Sosne G, Qiu P *et al*. 2007), which emphasize its therapeutic potential in the repair of damaged tissues or wound healing.

$T\beta_4$ is distributed ubiquitously in most tissues and cells, and is also known to concentrate highly at blood platelets (Huff T, Otto AM *et al*. 2002). These findings suggest that endogenous $T\beta_4$ likely promotes the healing of damaged tissues. Exogenous $T\beta_4$ was also reported to accelerate the tissue repair of damaged cardiac, corneal and dermal tissues (Bock-Marquette I,

Saxena A *et al*. 2004; Sosne G, Hafeez S *et al*. 2002; Philp D, Badamchian M *et al*. 2003), which demonstrates potential for clinical applications in wound healing.

Generally, wound healing in the oral cavity is known to occur more quickly and scar less than dermal tissue, which may be due to the elements in saliva and unique phenotype of oral fibroblasts (Lee HG and Eun HC. 1999; Häkkinen L, Uitto VJ *et al*. 2000; Lygoe KA, Norman JT *et al*. 2004). Despite the relatively fast wound healing, however, tissues damaged during periodontal and implant surgery are continuously challenged by bacterial infection in the oral cavity, necessitating meticulous maintenance of oral hygiene and additional plaque control. Prevention of bacterial contamination is even more important in the case of gingival graft surgery because a significant amount of tissue is lost at a palatal donor site. Autogenous gingival grafts are often accompanied by discomfort, pain and retarded tissue repair depending on a patient's condition. To avoid these post-operative problems, topical application of an antimicrobial treatment is recommended (Kozlovsky A, Artzi Z *et al*. 2007). Furthermore, several dressing materials, which are supposed to aid in tissue repair, are commonly applied to palatal wounds during the healing process (Hammad HM, Hammad MM *et al*. 2011;

Shanmugam M, Kumar TS *et al*. 2010). Accelerated palatal mucosa regeneration was reported to occur after treatment with a basic fibroblast growth factor impregnated in collagen-gelatin scaffold (Ayvazyan A, Morimoto N *et al*. 2011). Those studies showed that dressing materials or chemicals employed for treatment of dermal wound healing can also be effective for oral mucosa regeneration. Therefore, $T\beta_4$, which is known to enhance the regeneration of different types of tissue, is also expected to accelerate mucosal wound healing. In a previous study, $T\beta_4$ was documented to be a natural component of saliva. The concentrations in human saliva ranged from 0.2-3.6 μg/ml, varying with age and state of disease (Badamchian M, Damavandy AA *et al*. 2007). The $T\beta_4$ levels in gingival crevicular fluid from patients with periodontal disease were higher than that from healthy patients in the control group (Kwon E, Jacobs LC *et al*. 2013). Considering the various functions involved in wound healing, $T\beta_4$ in saliva or gingival crevicular fluid is thought to promote the repair of damaged oral tissues. The aim of this study was to evaluate the effect of $T\beta_4$ on palatal wound closure in a rat model. Because $T\beta_4$ already existed in saliva, the relatively high concentrations of $T\beta_4$ with carboxymethyl cellulose ointment (CMC) were applied. The effects of $T\beta_4$ on cell growth, adhesion and

migration of rat palatal (RP) cells were evaluated. Furthermore, the mRNA and protein expression of matrix metalloproteinase 2 (MMP2) and vascular endothelial growth factor (VEGF) were analyzed in $T\beta_4$-treated RP cells.

2.2 Materials and methods

Chemical reagents and cell culture. Cell culture medium and reagents were purchased from Gibco-BRL (Grand Island, NY, USA). Other experimental reagents were obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA), unless otherwise specified.

RP cells were obtained from the palatal tissues of 5-week old male Sprague-Dawley (SD) rats. Isolated palatal tissues were washed with phosphate buffered saline (PBS), minced into pieces, and then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotic solution (100 U/ml of penicillin-G and 100 μg/ml of streptomycin) at 37°C in a 5% CO_2 humidified incubator. After 20 days of culture with medium changes every 3 days, RP cells were collected and subcultured under the same conditions. Passages three through six were used for this study.

Cell Adhesion and proliferation assay. Adhesion and proliferation of RP cells on the polystyrene surface of culture plates were observed in the presence of $T\beta_4$. For the adhesion assay, 1×10^4 palatal cells were incubated in the wells of a 96-well plate with $T\beta_4$ at various concentrations ranging from 1-1000 ng/ml for 4 hours. The wells were gently washed three times with PBS, and the number of attached cells was quantified using 2-(2-methoxy-4-nitropenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetr azolium (WST-8, Dojindo Laboratories, Kumamoto, Japan). Cells were incubated in 100 μl of WST-8 solution for 1 hour at 37°C in humidified atmosphere (5% CO_2/95% air). The absorbance was measured at a wavelength of 450 nm using a plate reader (Sunrise, TECAN, Salzburg, Austria). To observe proliferation, palatal cells were treated with $T\beta_4$ for 24 hours, and the number of cells was measured by using WST-8.

In vitro wound closure assay. For the *in vitro* wound closure assay, a culture insert (ibidi GmbH, Martinsried, Germany) was employed to create a wound in cell culture. The culture insert was placed on a culture dish, and 70 μl of RP cell suspension (5×10^5 cells/ml) was added into both wells of the insert.

The RP cells were incubated at 37°C for 18 hours, and were serum-starved for 24 hours. After serum starvation, the culture insert was carefully removed, and the cells were exposed to $T\beta_4$ at various concentrations between 0-1000 ng/ml. The wound closure was observed and recorded at intervals under a phase contrast microscope (Olympus, Tokyo, Japan). This experiment was replicated three times.

mRNA expression analysis of MMP2 and VEGF. To investigate the effect of $T\beta_4$ on the mRNA expression of genes related to cell migration and angiogenesis, mRNA expression of MMP2 and VEGF was analyzed by real-time polymerase chain reaction (RT-PCR) assay. After serum starvation for 24 hours, palatal cells were treated with $T\beta_4$ for 6 and 24 hours and the total RNA was isolated using RNA extraction reagent (WelPrep Total RNA Isolation Reagent, Welgene Inc, Daegu, Korea). From the total RNA, cDNA was prepared using a cDNA synthesis kit (Power cDNA Synthesis Kit, iNtRON Biotechnology, Sungnam, Korea) and RT-PCR was performed in an ABI PRISM 7500 Sequence Detection System Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with 20 μl reaction volumes containing 10 μl SYBR premix Ex Taq (Takara Bio, Otsu, Japan), 0.4 μl ROX

Reference Dye II (Takara Bio), cDNA, and primers. The primers for gene amplification were as follows: VEGF sense, 5'-GAGTATATCTTCAAGCCGTCCTGT-3'; VEGF antisense, 5'-ATCTGCATAGTGACGTTGCTCTC-3'; MMP2 sense, 5'-CAGGGAATGAGTACTGGGTCTATT-3'; MMP2 antisense, 5'-ACTCCAGTTAAAGGCAGCATCTAC-3'; GAPDH sense, 5'-TGTGTCCGTCGTGGATCTGA-3'; GAPDH antisense, 5'-CCTGCTTCACCACCTTCTTGAT-3'. The PCR conditions were 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing at 60°C (34 seconds) for MMP2 and 63°C (34 seconds) for VEGF. All reactions were run in triplicate. Gene expression was evaluated based on the threshold cycle (CT value) and normalized to the amount of GAPDH transcript.

Western blot analysis. Western blot analysis was performed to examine the protein expression of MMP2 and VEGF in $T\beta_4$-treated palatal cells. After treatment with $T\beta_4$ for 6 hours, cells were centrifuged and re-suspended in an extraction buffer containing 50 mM Tris base-HCl (PH 8.0), 150 mM NaCl, 0.5% Triton-X 100, and 1 tablet of protease inhibitor cocktail (1

tablet/10 ml, Roche Applied Science, Mannheim, Germany) for 45 minutes on ice. Extracts containing equal amounts of protein were run on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The blots were incubated with rabbit polyclonal antibodies against VEGF, MMP2 or GAPDH in PBST (PBS containing 0.1% Tween 20) for 1.5 hour, washed three times with PBST, and then probed with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase. The antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA). The blots were developed using a chemiluminescence kit (WEST-ZOL plus Western Blot Detection System, iNtRON Biotechnology). Chemiluminescence was detected using the LAS 1000 Plus Luminescent Image Analyzer (Fuji PhotoFilm, Tokyo, Japan).

Rat palatal wound healing assay. The effect of $T\beta_4$ on wound healing of palatal tissue was investigated in a rat palatal wound model. Thirteen-week-old SD male rats, weighing 300-350 g, were used in this study. All animal experiments were performed under the control of the animal welfare committee of Seoul National University Institutional Animal Care and Use Committee. Under general anesthesia, punch wounds were

made on a central area of hard palate with a disposable 3 mm diameter biopsy punch (Kai Industries Ltd., Gifu, Japan), exposing a circular area of bare bone. The wound area was covered with 30% CMC ointment containing either 0 or 1 mg/ml $T\beta_4$. Six rats were used for each group. After the surgery, animals were fed a standard diet of pellets and water with enrofloxacin. The agents were re-applied at day 2 and 4 to reduce stress by anesthesia, and the rats were sacrificed at day 7. The maxillae were separated, and wound area was observed by stereoscopic microscope (Nikon, Tokyo, Japan) and by histological analysis. The wound areas in the microscopic images were calculated using CellSense Dimension 1.6 software (Olympus, Tokyo, Japan). The palatal specimens were fixed in 10% formalin for at least 24 hours, decalcified in Calci-Clear Rapid solution (National Diagnostics, Atlanta, GA, USA) for 35 hours, and processed further for histological analysis. Serial sections, 5 μm apart, were cut across the wound, perpendicular to the palatal midline at the widest diameter of the wound, and stained with haematoxylin and eosin. The sections were examined under a light microscope (Olympus), and the distance of wound margins in each section was measured with a calibrated ocular micrometer (Olympus).

Statistical analysis. Each experiment was performed in triplicate unless otherwise specified. Statistical analyses were performed by Student's *t*-test. *P*-value less than 0.05 is considered statistically significant.

2.3 Results

The effects of $T\beta_4$ on adhesion, proliferation and migration of palatal cells. To investigate the effects of $T\beta_4$ on the adhesion and proliferation of palatal cells, the cells were incubated for 4 and 24 hours in the presence of $T\beta_4$. At concentrations ranging from 1 to 1000 ng/ml, $T\beta_4$ did not exert any significant effects on the adhesion and proliferation of palatal cells (data not shown); however, cell migration was affected by $T\beta_4$ (Fig. 1). Untreated control cells did not exhibit any movement during 24 hours, possibly due to the serum-starved test conditions, but cell movement was observed when $T\beta_4$ was present at concentrations higher than 100 ng/ml. The migration effect of $T\beta_4$ appeared faster at the higher concentrations and cell motility was observed at 12 hours when incubated with 1000 ng/ml $T\beta_4$.

The effects of $T\beta_4$ on mRNA expression of MMP2 and VEGF genes. The

mRNA expression of MMP2 and VEGF was quantified to investigate the effects of $T\beta_4$ on cell migration and angiogenesis at molecular levels. As shown in Fig. 2 and Fig. 3, $T\beta_4$ enhanced gene expression of MMP2 and VEGF after 6 hours. The expression of MMP2 in the $T\beta_4$-treated group was significantly higher than in untreated cells. Although expression did not increase in a dose-dependent manner, the highest concentration of $T\beta_4$ (1000 ng/ml) led to the strongest induction of MMP2. $T\beta_4$ also enhanced the expression of the mRNA of VEGF gene in RP cells, inducing about 1.4-fold increase at 1000 ng/ml during 6 hours. A further increase of VEGF mRNA was obtained when exposed for 24 hours. However, the expression level of MMP2 gene was not maintained over the 24-hour period, and expression levels actually dropped below those of the untreated control (Fig. 4).

The effect of $T\beta_4$ on VEGF and MMP2 protein level. Protein expression of MMP2 and VEGF was determined by western blot analysis. RP cells were treated with $T\beta_4$ for 6 hours at various concentrations. The results show that $T\beta_4$ upregulated the expression of VEGF and MMP2 proteins in a dose-dependent manner (Fig. 5 and Fig. 6). The quantitative measurement of VEGF and MMP2 protein showed that treatment with $T\beta_4$ (1000 ng/ml)

significantly enhanced the level of VEGF protein by 2.1-fold, and enhanced the level of Pro-MMP2 and active MMP2 by 3.8- and 1.3-fold, respectively, when compared to the control (Fig. 5 and Fig. 6).

The effects of $T\beta_4$ on Palatal wound healing of rats. The wound healing effect of $T\beta_4$ was observed seven days after surgery, because the palatal wound gap was completely closed in less than two weeks in untreated rats (Fig. 7). As shown in Fig. 8, the wound area was not completely epithelized in either the control or the test groups. However, microscopically smaller wound areas were observed in the $T\beta_4$-treated test group, indicating that more advanced epithelization at the wound margin had taken place in $T\beta_4$-treated rats (Fig. 8A). The mean area of unepithelized surface in $T\beta_4$-treated rats was significantly smaller than that in control group (Fig. 8B) and histological examination also demonstrated an accelerated epithelization by $T\beta_4$ (Fig. 9A). The mean distance between the epithelized margin at the center of a palatal wound was about 1280 μm in $T\beta_4$-treated rats, while 1830 μm of wound remained unepithelized, on average, in the control group (Fig. 9B). Therefore, the present study has clearly demonstrated that $T\beta_4$ significantly accelerated the epithelization of rat palatal wounds.

2.4 Discussion

Wound healing is a series of events including inflammation, angiogenesis and reepithelization (Singer AJ and Clark RA. 1999). Because of the shorting comings of proteins such as high cost, easy degradation and hard preservation, peptide, which can overcome the shortcoming of the proteins and contains the active character of the protein, is regarded as effective alternatives. Fibrin-binding synthetic peptide significantly enhanced the cutaneous wound in diabetic rats (Chung JE, Kim YJ *et al*. 2013). Additionally, previous studies reported that $T\beta_4$ played a multi-functional role in wound repair processes, demonstrating a potential for the clinical use of $T\beta_4$ in wound care. The aim of this study was to investigate the feasibility of using $T\beta_4$ for oral wound repair. As shown in Fig. 1, $T\beta_4$ promoted palatal cell migration. Previously, MMPs were reported to be up-regulated during dermal wound repair in $T\beta_4$-treated rats (Philp D, Scheremeta B *et al*. 2006), and cell migration, promoted by $T\beta_4$, was blocked by a MMP inhibitor (Qiu P, Kurpakus-Wheater M *et al*. 2007). Those studies demonstrated a critical role of MMPs in $T\beta_4$-aided cell migration and wound healing. In this study, the expression of MMP2 was investigated, because mucosal fibroblasts are

known to mainly express MMP2 among MMP family related to wound healing (Mäkelä M, Salo T *et al*. 1994; Salo T, Mäkelä M *et al*. 1994). As shown in Fig. 2 and Fig. 5, the mRNA and protein expression of MMP2 was enhanced by $T\beta_4$, suggesting that the promotion of palatal cell migration by $T\beta_4$ was mediated by MMP2. The precise mechanism underlying the induction of MMP expression by $T\beta_4$ is not yet fully understood. However, previous studies suggesting $T\beta_4$ as a hypoxia responsible regulator (Moon EY, Im YS *et al*. 2010; Oh JM, Ryoo IJ *et al*. 2008) lead us to speculate that hypoxia-inducible factor (HIF)-1α may be involved in the $T\beta_4$-derived modulation of MMPs, because MMPs are known to be regulated by hypoxia or HIF-1α in other cell types (Ahn JK, Koh EM *et al*. 2008; Ben-Yosef Y, Miller A *et al*. 2005). Together with MMP2, VEGF, another main factor related to wound healing, is also a well-known target of HIF-1α. This study showed that VEGF was up-regulated by $T\beta_4$ (Fig. 3 and Fig. 6). Considering the ability of $T\beta_4$ to induce HIF-1α stabilization, it is assumed that HIF-1α was also involved in the enhanced expression of VEGF in the palatal cells.

In the animal model, $T\beta_4$ significantly accelerated the closure or reepithelization of palatal wounds (Fig. 8 and Fig. 9). The multi-functional effects of $T\beta_4$ on cell migration, angiogenesis and inflammation are all

thought to contribute the wound repair. Although $T\beta_4$ is an endogenous component of saliva, the present results showed that exogenous administration of $T\beta_4$ was effective for rapid healing of palatal wounds. In contrast to the applications in dermal wound healing, the administrated $T\beta_4$ can be easily washed away by saliva in the oral environment. Therefore, a more appropriate delivery system is expected to supply $T\beta_4$ continuously, which would accelerate healing processes. This study employed normal healthy rats to investigate the effects of $T\beta_4$. Further experiments elucidating the efficacy of $T\beta_4$ in regeneration-impaired animals due to diabetes or advanced age would be highly beneficial for determining the clinical potential of this treatment.

In conclusion, the cell migration of rat palatal cells was stimulated by $T\beta_4$. The protein and mRNA expression of MMP2 and VEGF were also enhanced by $T\beta_4$. Topical application of $T\beta_4$ greatly enhanced a palatal wound healing in rats. The present results suggest that $T\beta_4$ can be used for promotion of oral wound healing.

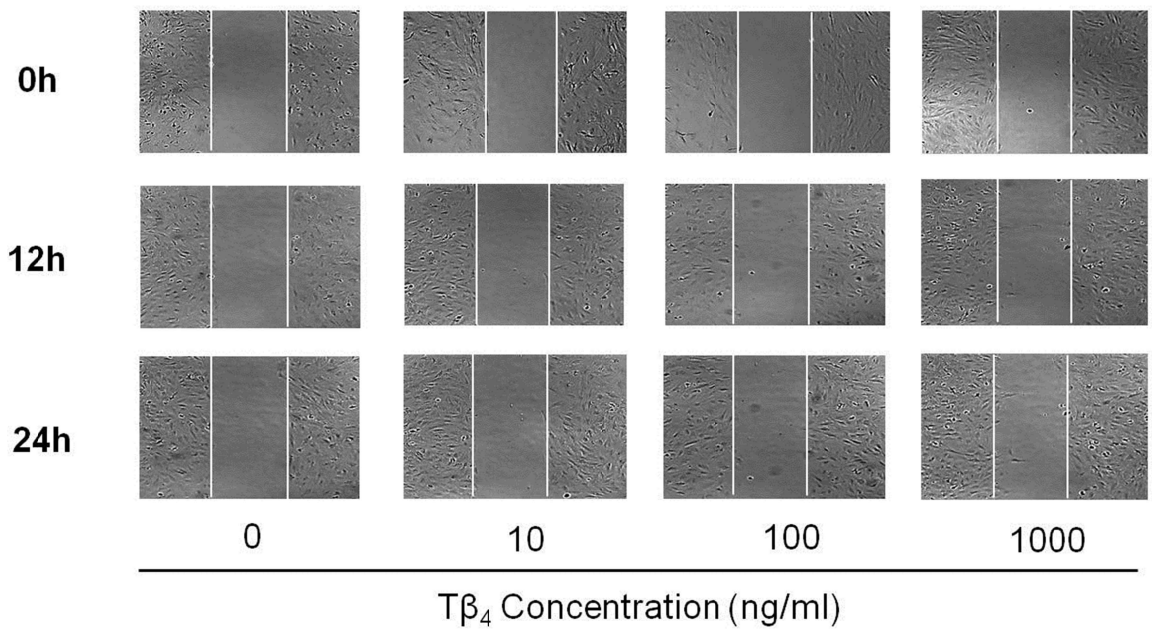


Figure 1. Effect of $T\beta_4$ on RP cell migration. Cells were incubated with $T\beta_4$ at various concentrations ranging from 0 to 1000 ng/ml, and cell movement was observed at 0, 12 and 24 hours with 40X magnification.

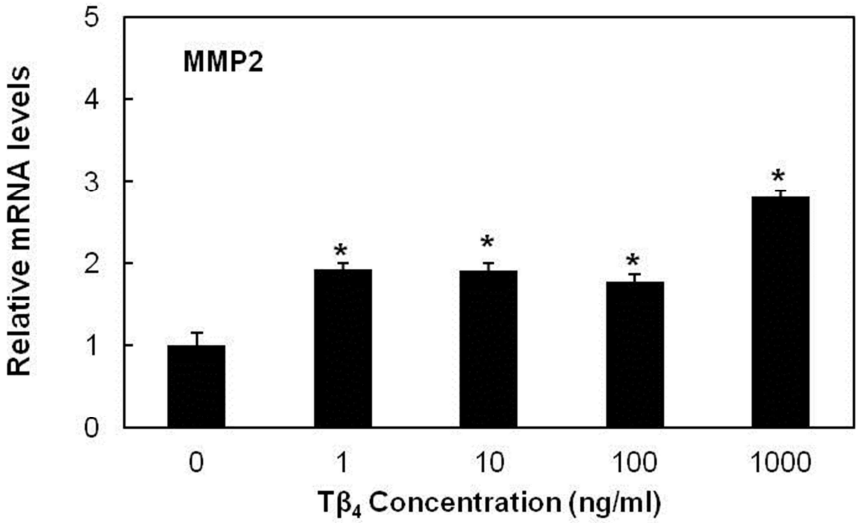


Figure 2. Effect of $T\beta_4$ on mRNA expression of MMP2 in RP cells. Cells were incubated with $T\beta_4$ for 6 hours. mRNA expression levels of each gene were normalized to GAPDH mRNA. Each value represents the mean ± SD of three independent experiments, and ∗ indicates a significant difference of mRNA levels between treated and untreated control cells (p<0.05).

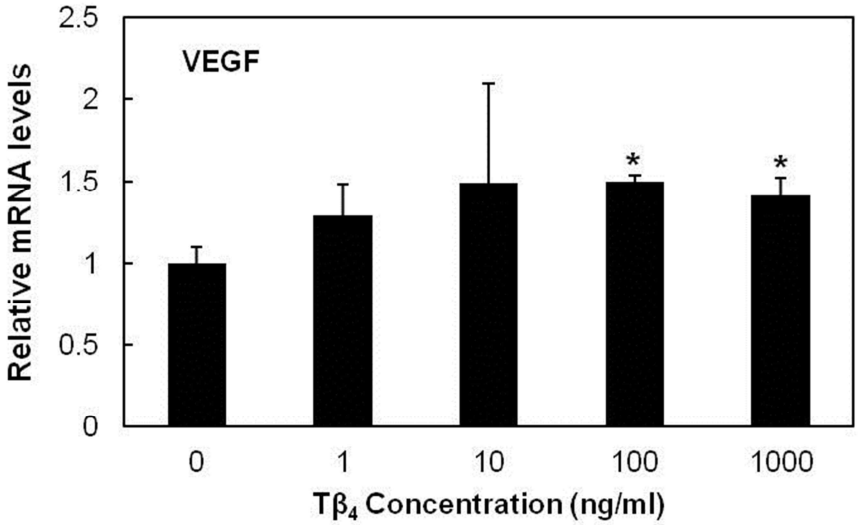


Figure 3. Effect of $T\beta_4$ on mRNA expression of VEGF in RP cells. Cells were incubated with $T\beta_4$ for 6 hours. mRNA expression levels of each gene were normalized to GAPDH mRNA. Each value represents the mean ± SD of three independent experiments, and ∗ indicates a significant difference of mRNA levels between treated and untreated control cells (p<0.05).

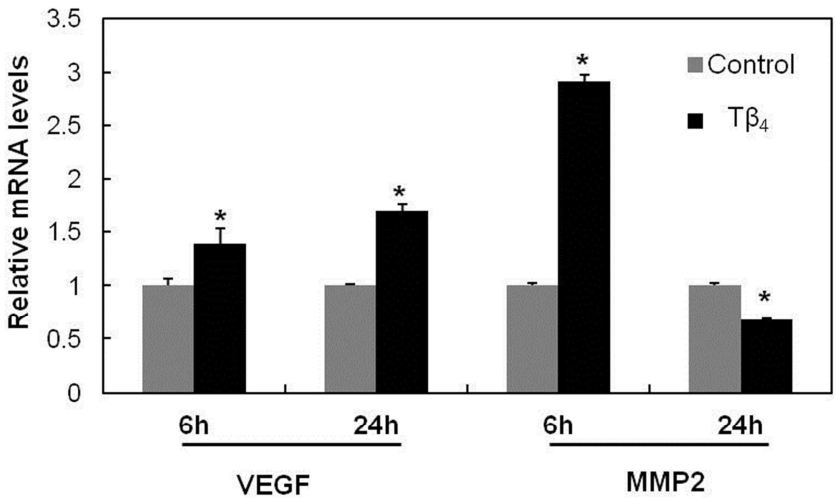


Figure 4. The mRNA Expression of MMP2 and VEGF genes in $T\beta_4$-treatd RP cells at different times. RP cells were treated with $T\beta_4$ (1 μg/ml) for 6 and 24 hours. Each value represents the mean ± SD of three independent experiments, and ∗ indicates that mRNA expression levels are significantly different from those of untreated control cells (p<0.05).

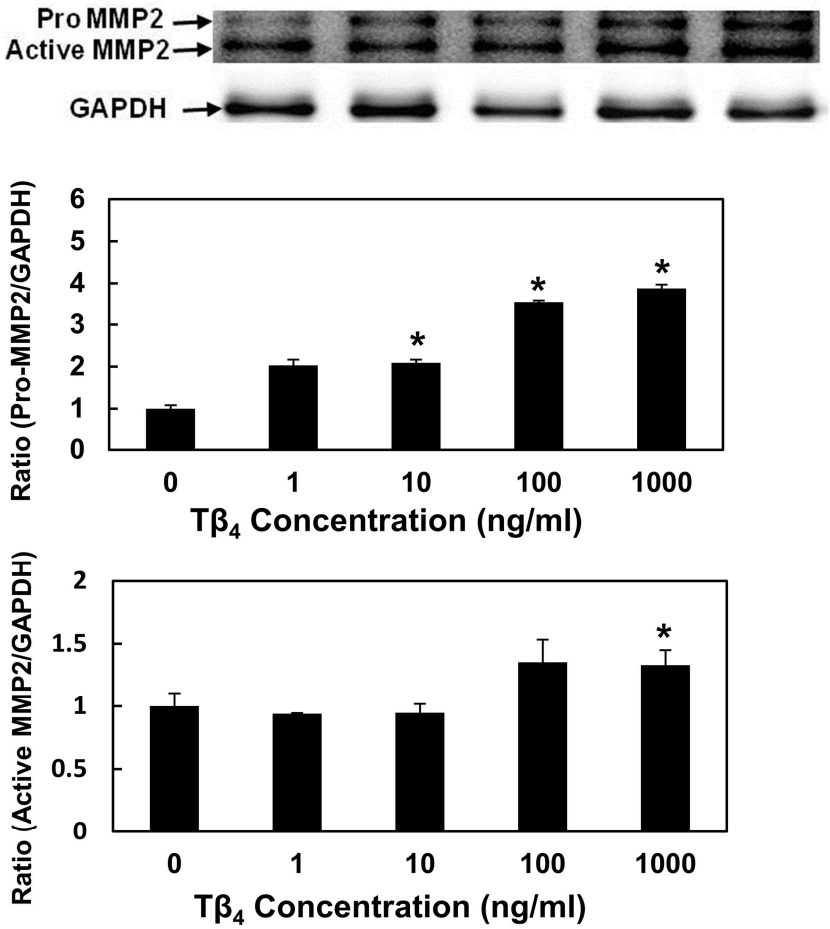


Figure 5. Effect of $T\beta_4$ on the protein expression of MMP2 . RP cells were treated with $T\beta_4$ for 6 hours. For quantitation of protein expression, the mean ± SD of three independent experiments was obtained. The blotting images are representatives of each experiment. ∗ indicates that protein expression levels are significantly different from those of untreated control cells (p<0.05).

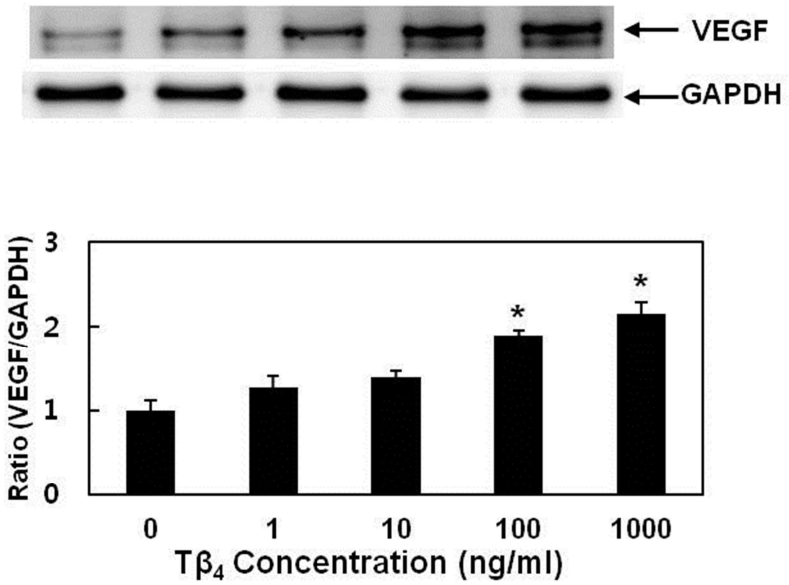


Figure 6. Effect of $T\beta_4$ on the protein expression of VEGF. RP cells were treated with $T\beta_4$ for 6 hours. For quantitation of protein expression, the mean ± SD of three independent experiments was obtained. The blotting images are representatives of each experiment. ∗ indicates that protein expression levels are significantly different from those of untreated control cells (p<0.05).

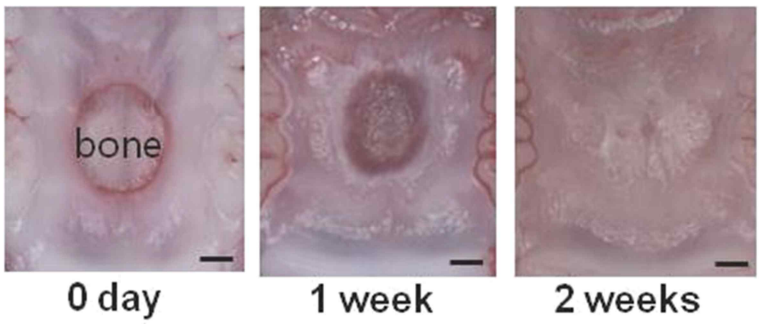
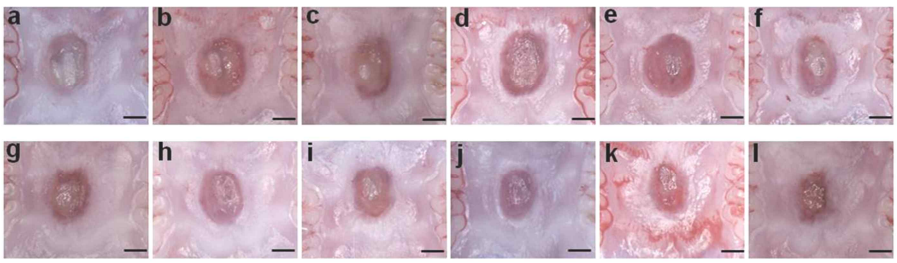


Figure 7. Effects of $T\beta_4$ on the healing of palatal wounds in untreated control group during 2 weeks. (Scale bar: 1 mm).

A



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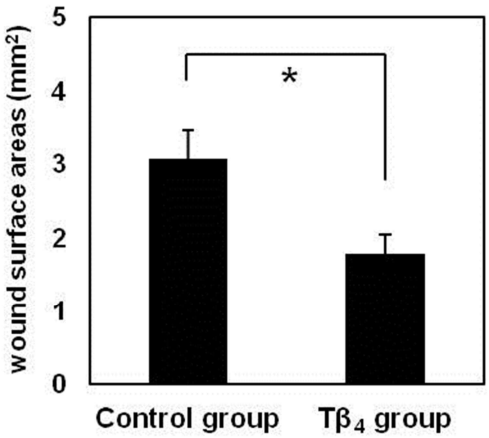
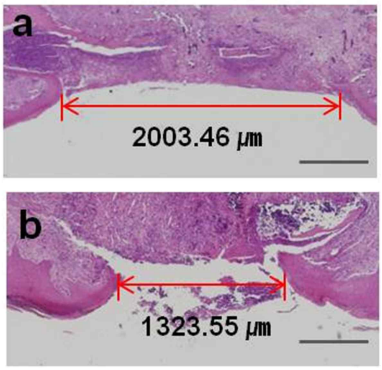


Figure 8. (A) Stereoscopic microscope images of the palatal wounds one week after surgery. $T\beta_4$ was topically applied to test groups (g-l), and 30% CMC (vehicle) was applied to control groups (a-f) (Scale bar: 1 mm). (B) Wound area calculated from the image of A. ∗ indicates that palatal wound surface areas of $T\beta_4$-treated groups was significantly different from that of the untreated control groups ($p< 0.05$).

A



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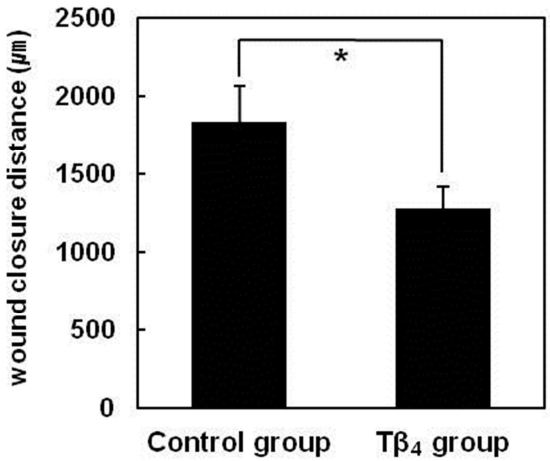

Figure 9. (A) Representative images of histological sections of palatal wounds. The sections of control (a) and $T\beta_4$-treated (b) wounds were stained with haemotoxylin and eosin (HE) (Scale bar: 500 μm). (B) Mean values of maximum palatal wound width calculated from the HE-stained sections. * indicates that palatal wound width of $T\beta_4$-treated groups was significantly different from that of the untreated control groups ($p<0.05$).

Chapter III.

Effects of dimethyloxalylglycine on wound healing of rat palatal mucosa

3.1 Introduction

Wound healing is a dynamic process including inflammation, angiogenesis and reepitheliazation (Shaw TJ and Martin P. 2009) Among them, angiogenesis is critical phase (Andrikopoulou E, Zhang X *et al*. 2011), which is defined as the formation of new blood vessels from established vessels (Johnson RB, Serio FG *et al*. 1999). Angiogenesis also contributes to the severity of the inflammation due to the pro-inflammatory cells transported by the newly formed vessels. In addition, angiogenesis is associated with the regeneration, which is impeded when inflammation come to the end (Agis H, Watzek G *et al*. 2012).

Vascular endothelial growth factor (VEGF) is a kind of representative marker which regulates the angiogenesis. VEGF can be up-regulated by several pathways in which hypoxia-inducible factor 1(HIF-1) pathway was

mostly highlighted. HIF-1 is comprised of oxygen inducible HIF-1α and constitutive HIF-1β subunits, which have an affiliation with the basic-helix-loop-helix PER-ARNT-SIM family (Wang GL, Jiang BH *et al*. 1995). Under normoxic conditions, specific proline residues located at the determined domain of HIF-1α, O_2-dependent degradation domain (ODD domain), was hydroxylated by prolyl hydroxylases (PHDs). Thereinto, prolyl hydroxylases 2 (PHD2) has the main function to control the HIF-1α expression (Takeda K, Ho VC *et al*. 2006)，and then with the cooperation of Fe^{2+}, 2-oxoglutarate and ascorbate (Hirsilä M, Koivunen P *et al*. 2003) to proceed the step binding to the von Hippel Lindau (VHL) complex, HIF-1α is degraded by ubiquitin-proteasome pathway (Yu F, White SB *et al*. 2001; Salceda S and Caro J. 1997; Sen CK. 2009). Under hypoxic conditions, PHDs' function was suppressed due to the absolute requirement with oxygen, allowing for HIF-1α stabilization and transcriptional activation of more than 60 target genes including VEGF, glucose transporter, etc (Andrikopoulou E, Zhang X *et al*. 2011). Therefore, HIF-1 pathway is the critical way to sense the oxygen tension. The control of HIF-1α can be benefit for angiogenesis and wound healing.

Previously, HIF-1α gene was transduced into bone marrow cells with

lentivirus vectors. Consequently, enhanced osteogenic and angiogenic capacity was found in HIF-1α transgenic bone marrow cells, providing a better treatment to cure large bone defects (Ding H, Gao YS et al. 2013) However, the over expression of HIF-1α throughout the transgenic cells' life and the danger of the lentivirus vectors should be seriously taken into account before clinical application. Indirectly, the knockout of PHD2 in keratinocytes leading to a faster wound healing in aged mice had been confirmed (Rezvani HR, Ali N *et al*. 2011).

Dimethyloxalylglycine (DMOG), with small molecular weight, is a cell-permeable unspecific inhibitor of PHD to stabilize the expression of HIF-1α in cells. DMOG can mimic the hypoxic conditions and prohibit the degradation of HIF-1α. Compared with the risk by lentivirus vectors, DMOG has the safe therapeutic ability for clinical use.

Botusan *et al* demonstrated the stabilization of HIF-1α by the PHD inhibitor DMOG to enhance the wound healing in diabetic mice (Botusan IR, Sunkari VG *et al*. 2008). Another study also indicated that DMOG decreased plasminogen activation in gingival fibroblast cell under normal and inflammatory conditions (Wehner C, Gruber R *et al*. 2014).

Previous study indicated the DMOG enhanced the production of VEGF in

periodontal fibroblast cells (Agis H, Watzek G *et al*. 2012). However, the effect of PHD inhibitor DMOG on the wound healing of the oral mucosa remains elusive. In the present study, the rat palatal wound model was used to evaluate the wound healing effect of DMOG. Additionally, the effect of DMOG on cell migration of rat palatal (RP) cells was also evaluated. Furthermore, the mRNA and protein expressions of VEGF were analyzed in DMOG-treated RP cells.

3.2 Materials and methods

Chemical reagents and cell culture. Cell culture medium and reagents were purchased from Gibco-BRL (Grand Island, NY, USA). Other experimental reagents were obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA), unless otherwise specified.

RP cells were obtained from the palatal tissues of 5-week old male Sprague-Dawley (SD) rats. Isolated palatal tissues were washed with phosphate buffered saline (PBS), minced into pieces, and then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotic solution (100 U/ml of penicillin-G and 100

μg/ml of streptomycin) at 37°C in a 5% CO_2 humidified incubator. After 20 days of culture with medium changes every 3 days, RP cells were collected and subcultured under the same conditions. Passages four through six were used for this study.

Cell viability assay. RP cells incubated at 80% confluent were trypsinized and subcultured at $0.8*10^5$ cells per ml in a 96-well plate and incubated at 37°C with 5% CO_2 in air for 24 hours, and then the medium was changed to the treatment medium with DMOG at different concentrations (0.1, 0.5, 1, 2, 4, 8, 10 mM). After treatment for 24 hours, the cell viabilities was quantified by 2-(2-methoxy-4-nitropenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8, Dojindo Laboratories, Kumamoto, Japan). Cells were incubated in 100 μl of WST-8 solution for 1 hour at 37°C in humidified atmosphere (5% CO_2/95% air). The absorbance was measured at a wavelength of 450 nm using a plate reader (Sunrise, TECAN, Salzburg, Austria). The optical density of untreated cells was designated as 100%. Each experiment was performed in triplicate at least.

In vitro wound closure assay. For the *in vitro* wound closure assay, a culture

insert (ibidi GmbH, Martinsried, Germany) was employed to create a wound in cell culture. The culture insert was placed on a culture dish, and 70 μl of RP cell suspension (5×10^5 cells/ml) was added into both wells of the insert. The RP cells were incubated at 37°C for 18 hours, and were serum-starved for 24 hours. After serum starvation, the culture insert was carefully removed, and the cells were exposed to DMOG at various concentrations between 0-50 μM. The wound closure was observed and recorded at intervals under a phase contrast microscope (Olympus, Tokyo, Japan). This experiment was replicated three times.

mRNA expression analysis of VEGF. To investigate the effect of DMOG on the mRNA expression of genes related to angiogenesis, mRNA expression of VEGF was analyzed by real-time polymerase chain reaction (RT-PCR) assay. After treatment with DMOG at different concentrations (0, 0.1, 0.5, 1, 2 mM) for 24 hours, the total RNA was isolated using RNA extraction reagent (WelPrep Total RNA Isolation Reagent, Welgene Inc, Daegu, Korea). From the total RNA, cDNA was prepared using a cDNA synthesis kit (Power cDNA Synthesis Kit, iNtRON Biotechnology, Sungnam, Korea) and RT-PCR was performed in an ABI PRISM 7500 Sequence Detection System

Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with 20 μl reaction volumes containing 10 μl SYBR premix Ex Taq (Takara Bio, Otsu, Japan), 0.4 μl ROX Reference Dye II (Takara Bio), cDNA, and primers. The primers for gene amplification were as follows: VEGF sense, 5'-GAGTATATCTTCAAGCCGTCCTGT-3'; VEGF antisense, 5'-ATCTGCATAGTGACGTTGCTCTC-3'; GAPDH sense, 5'-TGTGTCCGTCGTGGATCTGA-3'; GAPDH antisense, 5'-CCTGCTTCACCACCTTCTTGAT-3'. The PCR conditions were 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing at 63°C (34 seconds) for VEGF. All reactions were run in triplicate. Gene expression was evaluated based on the threshold cycle (CT value) and normalized to the amount of GAPDH transcript.

Western blot analysis. Western blot analysis was performed to examine the protein expression of HIF-1α and VEGF in DMOG-treated palatal cells. After treatment with DMOG at various concentrations (0, 0.1, 0.5, 1, 2 mM) for 24 hours, cells were centrifuged and re-suspended in an extraction buffer containing 50 mM Tris base-HCl (PH 7.5), 150 mM NaCl, 0.5% Triton-X 100, and 1 tablet of protease inhibitor cocktail (1 tablet/10 ml, Roche

Applied Science, Mannheim, Germany) for 45 minutes on ice. Extracts containing equal amounts of protein were run on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The blots were incubated with rabbit polyclonal antibodies against VEGF, HIF-1α or GAPDH in PBST (PBS containing 0.1% Tween 20) for 1.5 hour, washed three times with PBST, and then probed with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase. The antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA). The blots were developed using a chemiluminescence kit (WEST-ZOL plus Western Blot Detection System, iNtRON Biotechnology). Chemiluminescence was detected using the LAS 1000 Plus Luminescent Image Analyzer (Fuji PhotoFilm, Tokyo, Japan).

Rat palatal wound healing assay. The effect of DMOG on wound healing of palatal tissue was investigated in a rat palatal wound model. All rats were housed under specific-pathogen-free conditions at the experimental centre at the Dental School of Seoul National University. Thirteen-week-old SD male rats, weighing 300-350 g, were used in this study. All animal experiments were performed under the control of the animal welfare committee of Seoul

National University Institutional Animal Care and Use Committee. Under general anesthesia, punch wounds were made on a central area of hard palate with a disposable 3 mm diameter biopsy punch (Kai Industries Ltd., Gifu, Japan), exposing a circular area of bare bone. The wound area was covered with 20 mg/ml hyaluronic acid (HA) ointment containing 0, 0.5 or 1 mg/ml DMOG. Six rats were used for each group. After the surgery, animals were fed a standard diet of pellets and water with enrofloxacin. The agents were re-applied at day 2 and 4 to reduce stress by anesthesia, and the rats were sacrificed at day 7. The maxillae were separated, and wound area was observed by stereoscopic microscope (Nikon, Tokyo, Japan) and by histological analysis. The wound areas in the microscopic images were calculated using CellSense Dimension 1.6 software (Olympus, Tokyo, Japan). The palatal specimens were fixed in 10% formalin for at least 24 hours, decalcified in Calci-Clear Rapid solution (National Diagnostics, Atlanta, GA, USA) for 35 hours, and processed further for histological analysis. Serial sections, 5 μm apart, were cut across the wound, perpendicular to the palatal midline at the widest diameter of the wound, and stained with haematoxylin and eosin. The sections were examined under a light microscope (Olympus), and the distance of wound margins in each

section was measured with a calibrated ocular micrometer.

Statistical analysis. Each experiment was performed in triplicate unless otherwise specified. Statistical analyses were performed by Student's *t*-test. *P*-value less than 0.05 is considered statistically significant.

3.3 Results

The effects of DMOG on cell viability and migration of palatal cells. To investigate the effects of DMOG on the cell viability of palatal cells, the cells were incubated for 24 hours in the presence of DMOG. At concentrations ranging from 0 to 10 mM, DMOG did not exert any significant effects on viability of palatal cells from 0 to 2 mM, but the cytotoxicity was obviously demonstrated at the concentrations of DMOG higher than 2 mM, with about 60% cell viability at 4 mM and almost totally cell death at 8 and 10 mM. (Fig.11); however, cell migration was not affected by DMOG. Untreated control cells did not exhibit any movement during 24 hours, but cell movement was also not observed in DMOG treated cells, possibly due to the serum-starved test conditions. (Fig. 10)

The effects of DMOG on mRNA expression of VEGF genes. The mRNA expression of VEGF was quantified to investigate the effects of DMOG on cell angiogenesis at molecular levels. As shown in Fig. 12, DMOG enhanced gene expression of VEGF dose-dependently in RP cells. The expression of VEGF in the 2 mM DMOG-treated group was significantly 2.3-fold higher than that in untreated cells.

The effect of DMOG on VEGF and HIF-1α protein level. Protein expression of HIF-1α and VEGF was determined by western blot analysis. RP cells were treated with DMOG for 24 hours at various concentrations (0, 0.1, 0.5, 1, 2 mM). The results show that DMOG stabilized HIF-1α protein dose-dependently till 0.5 mM and maintained the ability till 2 mM (Fig. 13). DMOG up-regulated the expression of VEGF significantly at the higher concentrations (Fig. 14). The quantitative measurement of VEGF and HIF-1α proteins showed that treatment with DMOG maximally enhanced the level of VEGF protein by 3.7-fold , and enhanced the level of HIF-1α by 2.7-fold, respectively, when compared to the control (Fig. 13 and Fig. 14).

The effects of DMOG on Palatal wound healing of rats. The wound healing

effect of DMOG was observed seven days after surgery, because the palatal wound gap was completely closed in less than two weeks in untreated rats (Fig. 7). As shown in Fig.15, the wound area was not completely epithelized in either the control or the test groups. However, microscopically smaller wound areas were observed in the 1 mg/ml DMOG-treated test group (Fig. 16), indicating that more advanced epithelization at the wound margin had taken place in DMOG-treated rats. The mean wound closure distance in 1 mg/ml DMOG-treated rats was significantly smaller than that in control group (Fig. 17 and Fig. 18) while other DMOG-treated rats did not demonstrated a significantly accelerated epithelization compared with control group.

3.4 Discussion

In this study, the wound healing effect of DMOG on rat palatal mucosa was observed. As shown in Fig. 10, DMOG did not influence the migration of RP cells, possibly because of the No FBS status.

The cytotoxicity of DMOG at relatively higher concentrations was found (Fig. 11). The partly reason may be the cycle arrest by PHD inhibitors

(Scholzen T and Gerdes J. 2000). Other research also indicated that DMOG had the effect to protect mesenchymal stem cell against apoptosis and cell death (Liu XB, Wang JA *et al.* 2009). But in this research the highest concentration was 1 mM, which was non-toxical concentration in tested cells. This indicated the limitation of protection effect of DMOG.

Previously, it has been demonstrated that PHD inhibitors up-regulate the expression of VEGF in several different kinds of cells, including endothelial cells (Shafighi M, Qlariu R *et al*. 2011), epithelial cells (Asikainen TM, Ahmad A *et al*. 2005), gingival fibroblast and periodontal ligament (Agis H, Watzek G *et al.* 2012). In agreement with the results of those studies, the present study also confirmed the ability of DMOG on up-regulating the expression of VEGF (Fig. 12 and Fig. 14) in RP cells. DMOG also induced stabilization of HIF-1α protein dose-dependently till 0.5 mM and maintain the effects till 2 mM in the palatal cells (Fig. 13), which is in line with that VEGF is a target gene of HIF-1 (Forsythe JA, Jiang BH *et al*. 1996).

In the animal study, due to the remarkable angiogenesis effect of DMOG, topical application of DMOG at 1 mg/ml significantly decreased wound area of palatal (Fig. 15 and Fig. 16). In the histological study, the wound closure distances in 1 mg/ml DMOG-treated rats showed the significant difference

(Fig. 17 and Fig. 18). Possibly because of no obvious migration effect of DMOG on the palatal cells, the treatment with DMOG did not show notable influence on the wound distance. In addition, in the oral environment, the applied DMOG could be washed out by drinking water and secreting saliva, DMOG could not have a continuously effect on palatal wound. A proper delivery system, for example, collagen membrane, which can make the effect of DMOG continuously on the wound region, may accelerate the healing processes.

Prolyl hydroxylase inhibitors are not new for the clinic use. For instance, iron chelation therapy was applied in sickle cell disease (Vichinsky E, Bernaudin F *et al*. 2011) and myelodysplastic syndromes (Delforge M, Selleslag D *et al*. 2011). DMOG may be another candidate for agents promoting oral wound healing.

In conclusion, the mRNA expression of VEGF was enhanced by DMOG. The VEGF and HIF-1α protein expression were increased significantly by treatment with DMOG. Topical application of DMOG at the concentrations of 1 mg/ml significantly enhanced a palatal wound healing in rats. The present results suggest that DMOG can be used for promotion of oral wound healing.

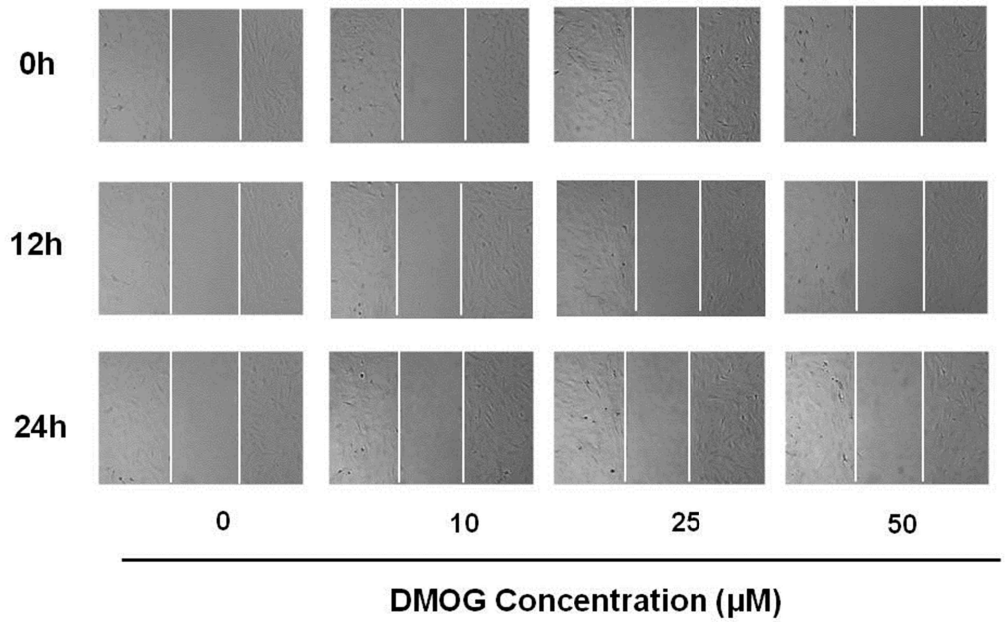

Figure 10. Effect of DMOG on RP cell migration. Cells were incubated with DMOG at various concentrations ranging from 0 to 50 μM, and cell movement was observed at 0, 12 and 24 hours with 40X magnification.

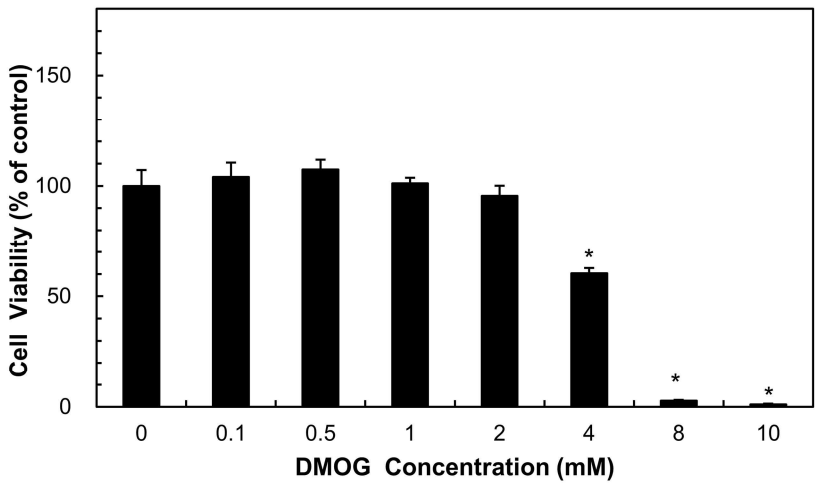

Figure 11. Effect of DMOG on the viability of RP cells. RP cells were treated with DMOG at the concentrations from 0 to 10 mM for 24 hours. Data and error bars indicate means±SD of three independent experiments. * demonstrates a statistically significant ($P < 0.05$) difference between control and treated cells.

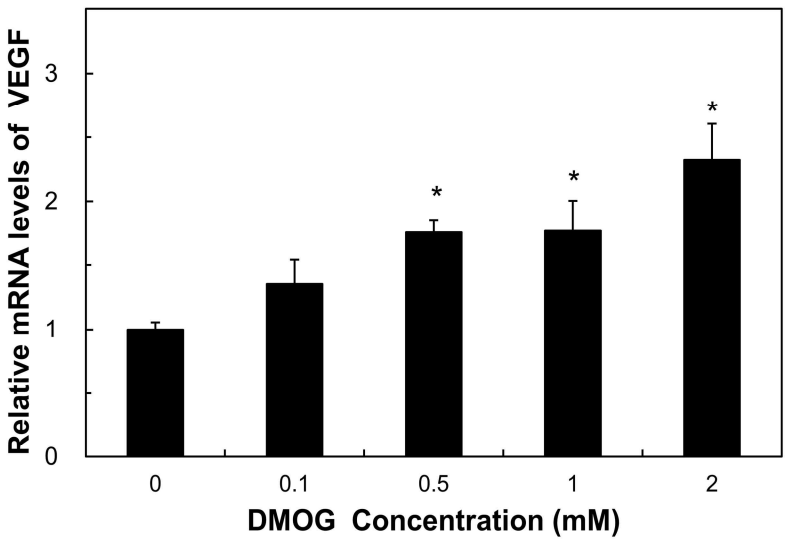

Figure 12. Effect of DMOG on the mRNA expression of VEGF gene. RP cells were treated with DMOG at the concentrations from 0 to 2 mM for 24 hours. Data and error bars indicate means ± SD of three independent experiments. * demonstrates that mRNA expression levels are significantly different from those of untreated control cells (P<0.05).

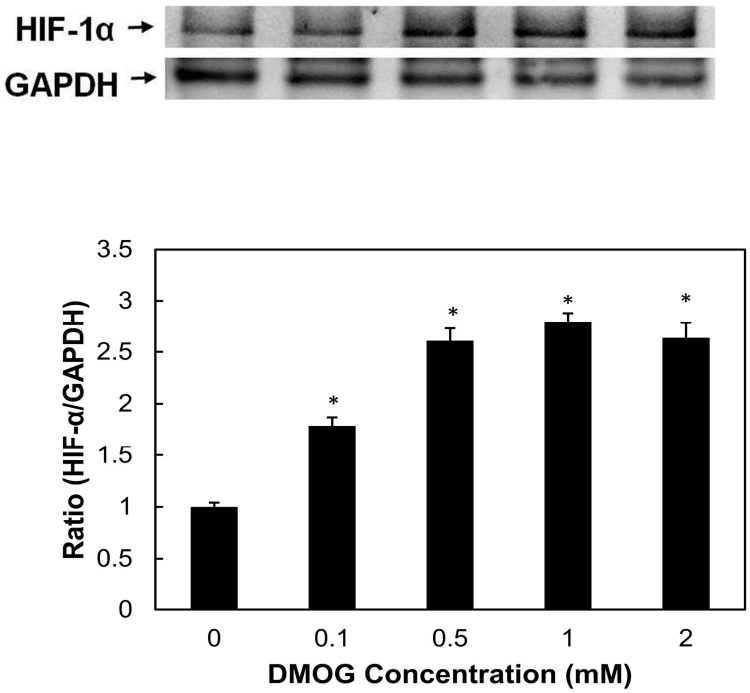

Figure 13. Effect of DMOG on the protein expression of HIF-1α. RP cells were treated with DMOG (0, 0.1, 0.5, 1, 2 mM) for 24 hours. For quantitation of protein expression, the mean ± SD of three independent experiments was obtained. The blotting images are representatives of each experiment. ∗ indicates that protein expression levels are significantly different from those of untreated control cells (p<0.05).

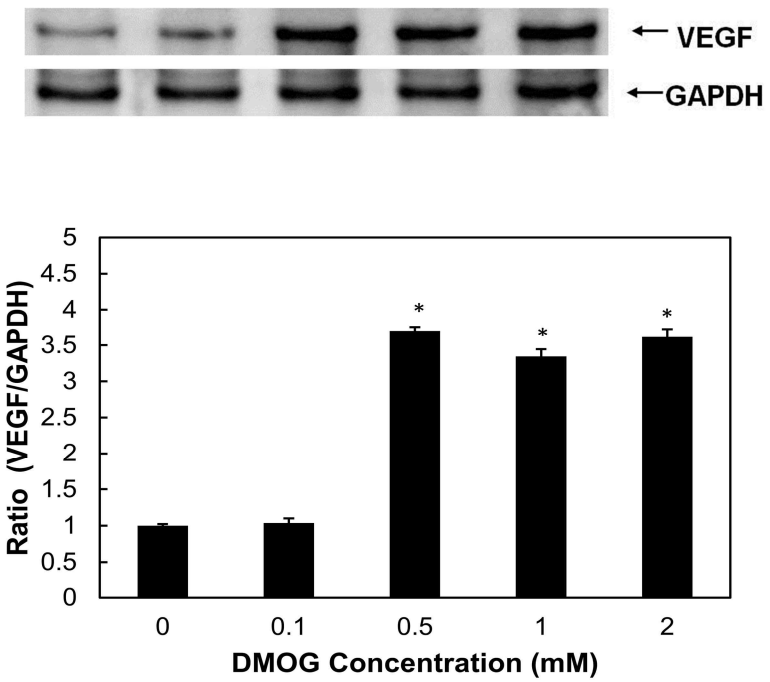

Figure 14. Effect of DMOG on the protein expression of VEGF. RP cells were treated with DMOG (0, 0.1, 0.5, 1, 2 mM) for 24 hours. For quantitation of protein expression, the mean ± SD of three independent experiments was obtained. The blotting images are representatives of each experiment. * indicates that protein expression levels are significantly different from those of untreated control cells (p<0.05).

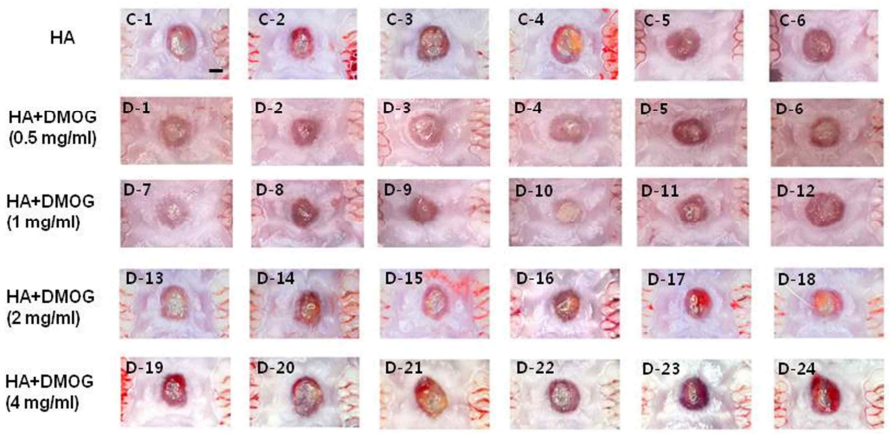

Figure 15. Effects of DMOG on the healing of palatal wounds. (A) Stereoscopic microscope images of the palatal wounds one week after surgery. DMOG was topically applied to test groups. 0.5 mg/ml was for D-1 to D-6, 1 mg/ml was for D-7 to D-12, 2 mg/ml was for D13 to D18 and 4 mg/ml was for D19 to D24), and 20 mg/ml HA (vehicle) was applied to control groups (C-1 to C-6) (Scale bar: 1 mm). (B) Wound area calculated from the image of Figure 15. ∗ indicates that palatal wound area of 1 mg/ml DMOG-treated groups was significantly different from that of the untreated control groups (p<0.05).

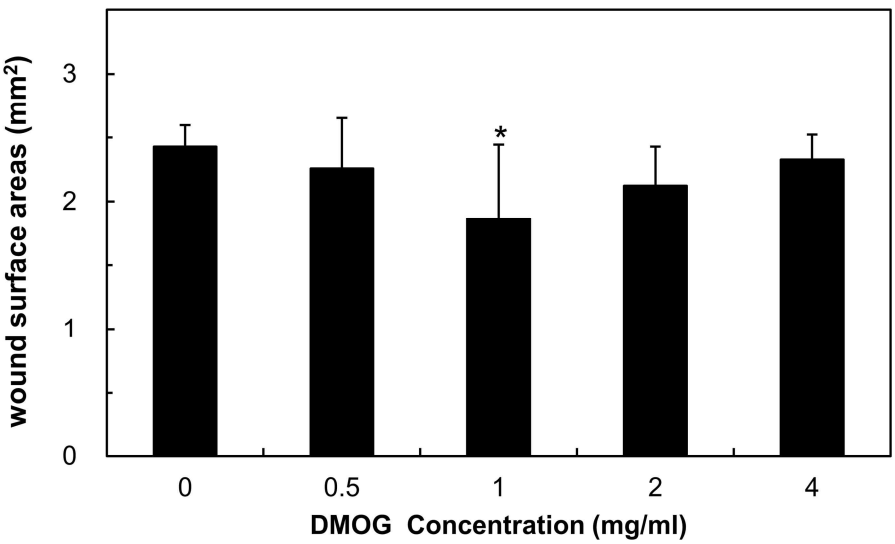

Figure 16. Effects of DMOG on the healing of palatal wounds. Wound area calculated from the image of Figure 15. ∗ indicates that palatal wound area of 1 mg/ml DMOG-treated groups was significantly different from that of the untreated control groups (p<0.05).

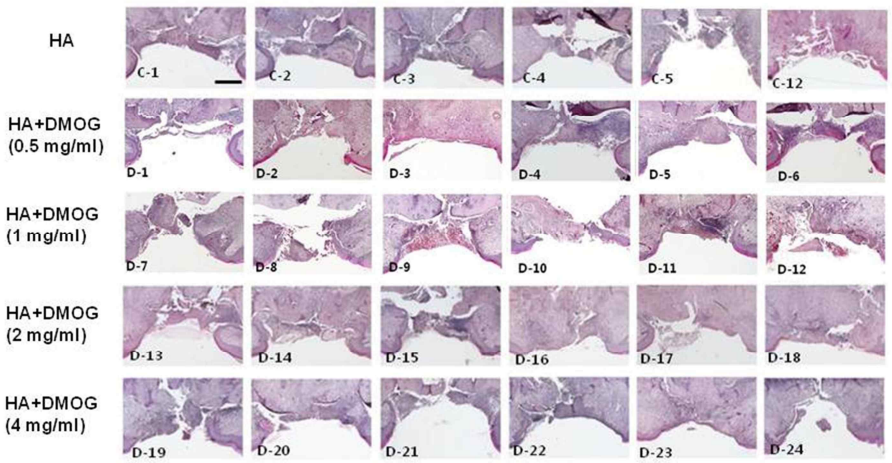

Figure 17. The sections of control and DMOG-treated wounds were stained with haemotoxylin and eosin (HE) (Scale bar: 500 μm).

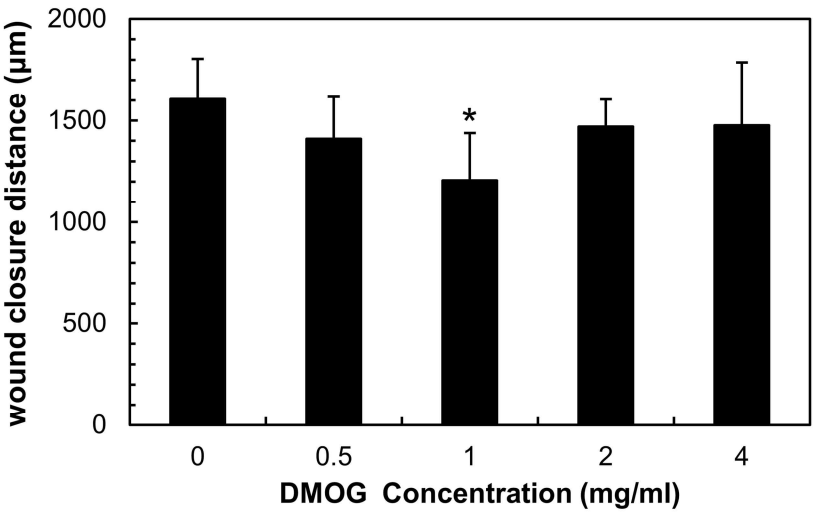

Figure 18. Mean values of maximum palatal wound width calculated from the HE-stained sections (Figure 17).

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Abstract in Korean

주정정

서울대학교 대학원

치의학과 치과생체재료과학 전공

목적: 43 개의 아미노산으로 구성 된 thymosin β_4 ($T\beta_4$)는 G-actin sequestering 단백질로 알려져 있으며, 혈관 생성, 세포사멸 억제, 항산화 및 항염증 등 다양한 생리활성 기능을 하는 것으로 보고되고 있다. Dimethyloxalylglycine (DMOG)는 저산소증 상태에서 안정화되는 전사조절 인자인 hypoxia inducible factor-1 alpha (HIF-1α) 분해를 억제하는 것으로 알려져 있으며, HIF-1α의 안정화는 혈관생성에 관여하는 단백질들의 발현을 유도하여 신생혈관 형성과 상처치유를 촉진할 수 있다. 본 연구에서는 $T\beta_4$와 DMOG 의 구개점막의 상처치유에 대한 효과를 조사하기 위하여, 구개점막세포의 세포이동 및 신생혈관생성 관련 유전자의 mRNA 및 단백질 발현과 백서구개에 형성된 창상치유에 대한 영향을 평가하였다.

재료 및 방법: Sprague-Dawley (SD) 백서에서 얻은 구개점막세포 (RPC)를 다양한 농도의 $T\beta_4$ 와 DMOG 로 처리하여 세포이동 분석을 시행하였으며, matrix metalloproteinase (MMP)와 vascular endothelial growth factor (VEGF) mRNA 및 단백질 발현 양을 측정하였다. 또한 HIF-1α 에 대한 DMOG 의 억제 효과를 확인하기 위하여 western blotting 법으로 HIF-1α 의 단백질 발현 양을 조사하였다. 백서 구개 점막 창상치유실험에서는 직경 3mm 의 생검펀치를 이용하여 SD 백서 구개 중앙 부분의 점막을 제거하고, 일 주 동안 $T\beta_4$ 및 DMOG 를 3 번 적용한 후 창상치유 정도를 육안 및 조직학적으로 관찰하였다.

결과 및 고찰: Serum starvation 환경하에서, $T\beta_4$ 는 고농도 (100 및 1000 ng/ml)에서 RPC 의 이동을 촉진하였지만 DMOG 는 RPC 의 이동에 영향을 주지 않았다. 또한 $T\beta_4$ 는 MMP2 및 VEGF 의 mRNA 및 단백질 발현을 증가시켰다. $T\beta_4$ 는 6 시간에서 MMP2 mRNA 발현을 증가시켰으나, 24 시간에서는 mRNA 발현이 감소하였다. DMOG 는 농도의존적으로 VEGF 의 mRNA 와 단백질 발현을 촉진하였다. 또한 DMOG 는 RPC 내 HIF-1α stabilization 을 유도하였다. 백서 구개 창상치유 실험에서 1 mg/ml 의 $T\beta_4$ 및

DMOG는 구개 창상치유를 통계적으로 유의하게 촉진하였다.

결 론: 본 연구 결과 $T\beta_4$ 와 DMOG 모두 백서구개 창상치유에 대한 촉진효과를 보여, $T\beta_4$ 및 DMOG 가 구개점막 상처 치유에 대한 유용한 물질로 제안할 수 있다.

주요어: 창상치유; 구강 점막; thymosin β_4; dimethyloxalylglycine
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